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Exploiting Catalytic Promiscuity for Biocatalysis

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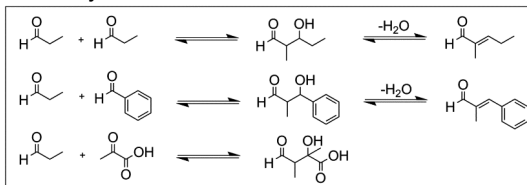
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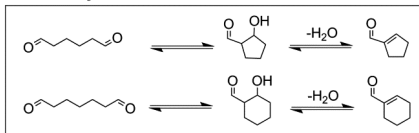
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Inter- and Intramolecular Aldol Reactions Catalyzed by a Highly Promiscuous Proline-based Tautomerase

4-OT-catalyzed intermolecular aldol reactions



4-OT-catalyzed intramolecular aldol reactions



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Abstract

The enzyme 4-oxalocrotonate tautomerase (4-OT), which in nature catalyzes an enol-keto tautomerization step as part of a catabolic pathway for aromatic hydrocarbons, was demonstrated to promiscuously catalyze different types of aldol reactions, namely intermolecular self-condensation, intermolecular cross-coupling and intramolecular cyclization. Mutation of an active site residue (F50A) enhances 4-OT's aldolase activities. This catalytic promiscuity of 4-OT could be exploited as starting point to create tailor-made aldolases for challenging cross-aldolizations.

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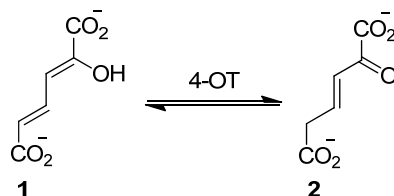
ANOTHER SHOWCASE OF 4-OT: THE ALDOLASE ACTIVITY

Introduction

The aldol reaction is one of the most fundamental carbon-carbon bond-forming reactions in organic chemistry. The reaction is widely used in the production of various chemicals including pharmaceutical compounds.^[1–5] Several methodologies have been employed to carry out aldol reactions including acid-, base-, organo-, metal-, and biocatalysis.^[6–8] Among them, biocatalysis receives considerable attention due to the fact that biocatalytic aldol reactions^[2,3,9] may be performed under environmentally friendly conditions.^[6] Aldolases, a specific group of lyases, constitute a class of enzymes that catalyze aldol reactions as their natural activity.^[9–12] Although aldolases can catalyze the cross-aldolization of a ketone and an aldehyde, enzyme-catalyzed cross-aldolizations of two aldehydes are extremely rare.^[9–20] Besides enzymes with natural aldolase activity (i.e., aldolases), there are few enzymes that promiscuously catalyze aldol reactions.^[3,21–23]

An enzyme that has recently attracted attention concerning its various promiscuous activities, including the cross-aldolization of two aldehydes, is the enzyme 4-oxalocrotonate tautomerase (4-OT).^[24–30] It is a member of the tautomerase superfamily, a group of homologous proteins having a β - α - β structural fold and a catalytic amino-terminal proline (Pro-1) in common.^[31–33] 4-OT takes part in a catabolic pathway for aromatic hydrocarbons in *Pseudomonas putida* mt-2, where it catalyzes the conversion of 2-hydroxyhexa-2,4-dienedioate (**1**, Scheme 1) into 2-oxohexa-3-enedioate (**2**).^[34,35] In this tautomerization reaction, Pro-1 acts as a general base ($\text{p}K_{\text{a}}$ of Pro-1 ~ 6.4) abstracting the 2-hydroxy proton of **1** and transferring it to the C5-position to give **2** (Scheme 1).^[36]

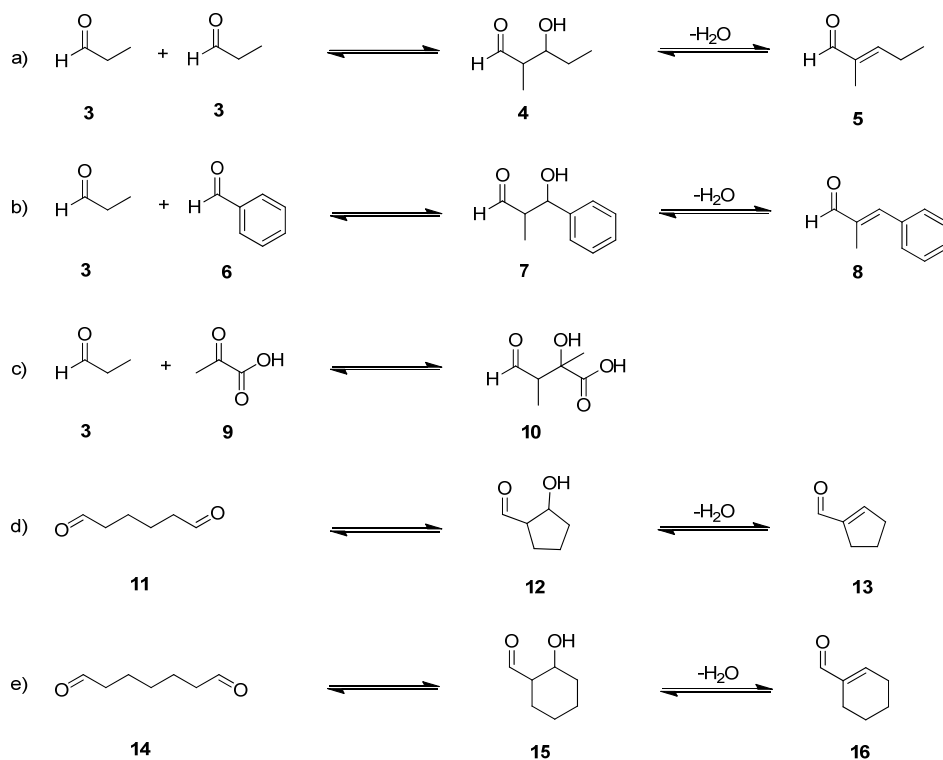
We have recently reported that 4-OT promiscuously catalyzes various C-C bond-forming reactions,^[24–30,37,38] including the aldol condensation of acetaldehyde and benzaldehyde to yield cinnamaldehyde.^[25,28] In this aldol condensation, 4-OT catalyzes both the aldol-coupling step and the subsequent dehydration step.^[25,28] NaCNBH_3 trapping, mass spectrometry, and X-ray crystallography^[39] experiments strongly suggest a mechanism in which Pro-1 functions as a nucleophile and reacts with the carbonyl functionality of acetaldehyde to form an enamine intermediate.^[25,28] This nucleophilic species reacts with benzaldehyde in an aldol addition. However, a mechanism in which Pro-1 functions as a catalytic base and that involves the formation of a reactive enolate intermediate cannot be excluded. Mechanism-inspired engineering provided an active site mutant (F50A) with strongly enhanced aldol condensation activity (600-fold in terms of $k_{\text{cat}}/K_{\text{m}}$).^[28]



Scheme 1. Natural proton-transfer reaction catalyzed by 4-OT.

In the present study we show that WT 4-OT and the 4-OT F50A mutant accept various carbonyl compounds as substrates for both inter- and intramolecular aldol reactions (Scheme 2). Interestingly, WT 4-OT and the 4-OT F50A mutant were also found to be capable of catalyzing proton-deuterium (H-D) exchange at acidic positions of carbonyl substrates, an activity in which residue Pro-1 plays a crucial catalytic role. This H-D exchange activity indicates that the active site of 4-OT can deprotonate carbonyl compounds, thereby providing additional evidence for a mechanism for the 4-OT catalyzed aldol reactions in which the aldehyde substrate is activated for nucleophilic addition via Pro-1 dependent formation of an enolate or enamine intermediate.

To investigate the substrate scope of wide type (WT) 4-OT and the 4-OT F50A mutant, these enzymes were purified to near homogeneity and examined for their ability to promiscuously catalyze the a) self-condensation of propanal (**3**), b) cross-coupling of propanal (**3**) and benzaldehyde (**6**), c) cross-coupling of propanal (**3**) and pyruvate (**9**), d) intramolecular cyclization of hexanedial (**11**), and e) intramolecular cyclization of heptanedial (**14**, Scheme 2). In addition, the P1A mutant of 4-OT was purified and tested as biocatalyst for these reactions in order to confirm the importance of the Pro-1 residue for catalysis.



Scheme 2. Promiscuous aldol reactions catalyzed by 4-OT; a) self-condensation of propanal (**3**); b) cross-coupling of propanal (**3**) and benzaldehyde (**6**); c) cross-coupling of propanal (**3**) and pyruvate (**9**); d) intramolecular cyclization of hexanedial (**11**) and e) intramolecular cyclization of heptanedial (**14**).

In separate experiments, WT 4-OT, 4-OT F50A, and 4-OT P1A (0.15 mM) were incubated with **3** (50 mM) in NaH_2PO_4 buffer (20 mM, pH 7.3) and reaction progress was monitored by UV spectroscopy. The UV spectra of the reaction mixture incubated with the 4-OT F50A mutant (Figure 1A) show an increase in absorbance at 234 nm in course of time which corresponds to the formation of product **5** ($\lambda_{\text{max},5} = 234 \text{ nm}$). A slight increase in absorbance at 234 nm was observed during the assay with WT 4-OT (Figure 1B). During the assay with the 4-OT P1A mutant, a negligible increase in absorbance at 234 nm was observed after 20 h (Figure 1C) while no change in absorbance was observed for the reaction mixture without enzyme (Figure 1D). The UV spectroscopic data strongly suggest that the WT 4-OT and 4-OT F50A enzymes catalyze the self-condensation of **3**, though the activity of WT 4-OT is relatively low. These results also indicate that the active site mutation of Phe-50 to Ala strongly increases the activity of 4-OT for self-condensation of **3**.

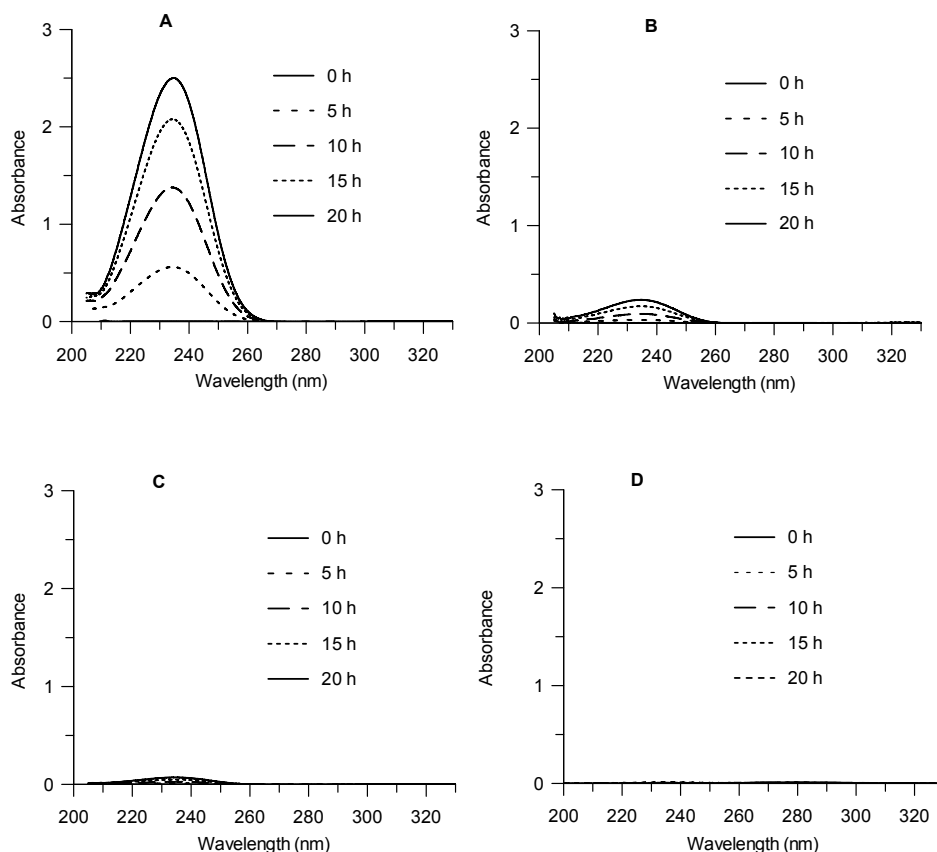


Figure 1. UV spectra showing the self-condensation of propanal (**3**) and the formation of 2-methyl-2-pentenal (**5**, $\lambda_{\text{max},5} = 234 \text{ nm}$). Propanal (**3**, 50 mM) in 20 mM NaH_2PO_4 buffer (pH 7.3) was incubated with A) 4-OT F50A mutant, B) WT 4-OT, C) 4-OT P1A mutant, D) no enzyme (control sample).

The self-condensation of **3** catalyzed by WT 4-OT, 4-OT F50A or 4-OT P1A was also monitored by ^1H NMR spectroscopy to verify that the product of the reaction is **5**. In separate experiments, WT 4-OT, 4-OT F50A, and 4-OT P1A (1.0 mol% relative to **3**), which were redissolved in NaD_2PO_4 buffer (20 mM, pD 7.6), were incubated with **3** (30 mM) in NaD_2PO_4 (20 mM, pD 7.6). A control experiment (i.e., reaction mixture without enzyme) containing **3** (30 mM) in NaD_2PO_4 buffer (20 mM, pD 7.6) was also performed. Our first observation was that during all experiments, including the control experiment, an equilibrium between the hydrated (63%) and unhydrated (37%) form of **3** was reached in the time between mixing all sample components and recording the first ^1H NMR spectrum of the reaction mixture (≤ 5 min) (Figure 2B, Scheme S2). Therefore, we could not distinguish whether the enzymes accelerate reaching this equilibrium or not. ^1H NMR spectroscopic signals of the unhydrated (i.e., propanal: 1.06, 2.57 and 9.69 ppm) and hydrated (i.e., propane-1,1-diol- d_2 : 0.92, 1.59 and 4.96 ppm) form of **3** are shown in Figure 2B.

We noticed another interesting phenomenon while analyzing the ^1H NMR spectra. The acidic protons of substrate **3**, which are located at the α -position (C2, marked with g in spectrum B, Figure 2) of the carbonyl group, were exchanged with deuterium in the reaction mixtures incubated with WT 4-OT and 4-OT F50A, respectively (Figures 2D and E). The exchange most likely only takes place at C2 of the unhydrated form of **3** (i.e., propanal) and not at C2 of the hydrated form (i.e., propane-1,1-diol- d_2) since the protons at C2 of the latter are not acidic (Scheme S2). However, since the rate for reaching equilibrium between unhydrated and hydrated form is relatively high compared to the rate of H-D exchange, the vanishing of signals g (protons at C2 of unhydrated form) and j (protons at C2 of hydrated form) was witnessed in equal proportion (Spectra D and E in Figure 2 and Scheme S2). The ^1H NMR data showed that the H-D exchange was faster in the presence of WT 4-OT ($\sim 90\%$ in 24 h) as compared to the H-D exchange in the presence of the 4-OT F50A mutant ($\sim 65\%$ in 24 h). A relatively low rate of H-D exchange was found for the control sample without enzyme (6%, 24 h) and 4-OT P1A (5%, 24 h), indicating that the H-D exchange is enzyme-catalyzed and that the Pro-1 residue is involved in catalysis (Figures 2C and F).

Whitman and co-workers have reported that the replacement of residue Phe-50 by an alanine makes the active site of 4-OT more accessible to the external aqueous environment, causing an increase in the local dielectric constant and in the pK_a of Pro-1 (from 6.4 to 7.3).^[40] Most likely, the F50A mutation makes the active site of 4-OT more amenable to hydrolysis of the covalent enzyme-product complex, the presumed rate-limiting step in which the aldol condensation product is released from Pro-1, and therefore a better catalyst for aldol condensation as we have reported previously.^[28] In contrast, the higher pK_a of Pro-1 of 4-OT F50A as compared to that of Pro-1 of WT 4-OT may result in a lower concentration of enzyme with Pro-1 in the correct protonation state for catalysis and thus in the lower rate of H-D exchange observed with 4-OT F50A as compared to WT 4-OT.^[33] The proposed mechanisms of proton-deuterium exchange during which Pro-1 either acts as a base or nucleophile are visualized in Scheme S3.

Finally, the ^1H NMR spectra (Figure 2, spectra C-F) were analyzed as well for the identification of product **5**. As shown in Figures 2D and 2E, the signals corresponding to product **5** were identified in ^1H NMR spectra of the reaction mixtures incubated with WT 4-OT and the 4-OT F50A mutant for 4 d. However, we observed that the integration of signal **b** of **5** in ^1H NMR spectra of the sample containing 4-OT F50A was lower than expected. The latter signal was missing in the ^1H NMR spectra of the reaction mixture incubated with WT 4-OT (vide infra for explanation) (compare Figures 2A, D, and E). During all experiments, no ^1H NMR signals corresponding to the hydrated form of **5** (conversion of the carbonyl moiety into diol), nor of the aldol coupling product **4** or its hydrated form,

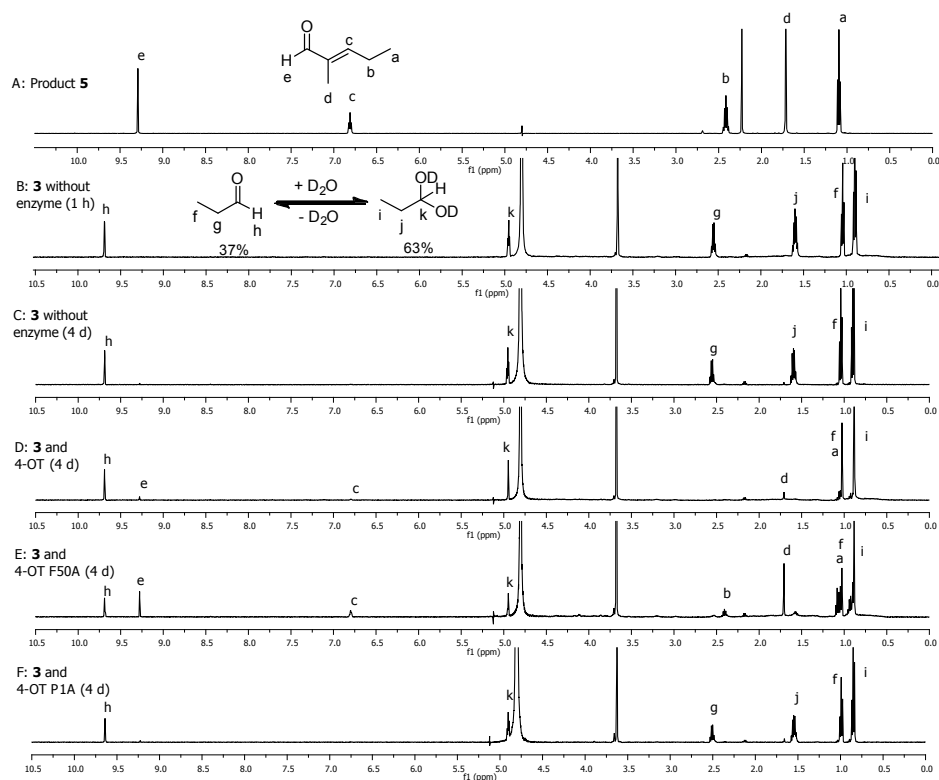


Figure 2. Stack plot of ^1H NMR spectra. A) ^1H NMR spectrum of an authentic sample of **5** (2-methyl-2-pentenal). Signals of **5** are marked with a,b,c,d, and e; B) Propanal (**3**) incubated in 20 mM NaD_2PO_4 buffer at pD 7.6 for 1 h. Equilibrium is reached between unhydrated and hydrated forms of **3** and signals are marked with f,g,h and i,j,k, respectively; C) Propanal (**3**) incubated in 20 mM NaD_2PO_4 buffer at pD 7.6 for 4 d. Signals of unhydrated and hydrated forms of **3** are marked with f,g,h and i,j,k, respectively; D) Propanal (**3**) incubated in 20 mM NaD_2PO_4 buffer at pD 7.6 with WT 4-OT for 4 d. Acidic protons of **3** (marked with g and j in spectrum B) are completely exchanged with deuterium. As a result, methyl signals f and i have changed from triplets to singlets (compare with spectrum B). Signals of **5** are marked with a,c,d, and e; E) Propanal (**3**) incubated in 20 mM NaD_2PO_4 buffer at pD 7.6 with 4-OT F50A for 4 d. Signals of **5** are marked with a,b,c,d, and e. F) Propanal (**3**) incubated in 20 mM NaD_2PO_4 buffer at pD 7.6 with 4-OT P1A for 4 d.

were detected over the entire course of reaction. The yield^[41] of **5** in the reaction mixture incubated with 4-OT F50A reached to ~27% after 4 d, whereas the yield of **5** in the reaction mixture incubated with WT 4-OT was ~5% after 4 d (Figures 2D and 2E). Only 1 and 2% yield of **5** was detected in the control and 4-OT P1A sample, respectively, after 4 d (Figures 2C and F). Incubation of **3** with synthetic 4-OT WT (prepared by total chemical synthesis)^[42] also led to the formation of **5** (Figure S1, spectrum D). This experiment confirms that 4-OT is responsible for catalysis and rules out the possibility that catalysis was effected by a contaminating enzyme from the expression strain.

In conclusion up to this point, the ¹H NMR data verified that WT 4-OT and 4-OT F50A catalyze the self-condensation of **3** and confirmed the essential role of Pro-1 in catalysis.^[43] In addition, a single site mutation of Phe-50 to Ala improves this activity of 4-OT. ¹H NMR spectroscopy also revealed that H-D exchange at the acidic α -position (C2) of the carbonyl functionality of **3** is catalyzed by both WT 4-OT and the 4-OT F50A mutant. Control experiments proved that the Pro-1 residue is essential for catalysis of the H-D exchange of **3**. Although not quantified, it became apparent that the rate of H-D exchange within **3** was higher than the rate of self-condensation of **3** in the presence of WT 4-OT or 4-OT F50A. This means that the majority of acidic protons of **3** (at C2) were exchanged with deuterium (relatively fast reaction) before **3** underwent self-condensation (relatively slow reaction) to yield product **5** (Scheme S2). During conversion, the C2 atoms of the two molecules of **3** become C2 and C4 of **5**, respectively. As a result, we noticed that signal b (C4) of **5** was missing in the ¹H NMR spectra of the reaction mixture incubated by WT 4-OT and this signal had lower integration area than expected in the ¹H NMR spectra of the 4-OT F50A sample. Besides these enzyme-catalyzed reactions, the ¹H NMR data also indicated that an equilibrium between **3** and its hydrated form was reached within 5 min. In fact, this equilibrium was reached before any H-D exchange was observed. Hydration of **3** may limit the yield of **5**. This is due to the fact that when compound **3** is present in its hydrated form its carbonyl functionality is converted into a diol, meaning that only the unhydrated form of **3** can react with Pro-1 to form an enolate or enamine intermediate to proceed with the reaction (Schemes S2 and S3). Although the rates for reaching equilibrium between hydrated and unhydrated **3** are relatively high, only 37% of substrate **3** is directly available for catalysis.

Next, we investigated the activity of WT 4-OT and the 4-OT F50A mutant for the cross-coupling of **3** and **6** (Scheme 2). In separate experiments, WT 4-OT, 4-OT F50A, and 4-OT P1A (0.15 mM) were incubated with propanal (50 mM) and benzaldehyde (2 mM) in NaH₂PO₄ buffer (20 mM, pH 7.3), and the reactions were monitored by UV spectroscopy for 20 h. Figure 3A shows the UV spectra of the reaction mixture incubated with the 4-OT F50A mutant. A decrease in absorbance at 250 nm was observed over a period of 5 h indicating the depletion of **6** ($\lambda_{\text{max},6} = 250$ nm). However, after 5 h the absorbance at 250 nm increased again and a new peak with a maximum at 241 nm appeared which may indicate the formation of **5** ($\lambda_{\text{max},5} = 234$ nm) by self-condensation of **3**. Neither formation of dehydrated aldol product **8** ($\lambda_{\text{max},8} = 288$ nm) was observed under these conditions within the time frame of 20 h, nor formation of aldol coupling product **7** as it most likely does

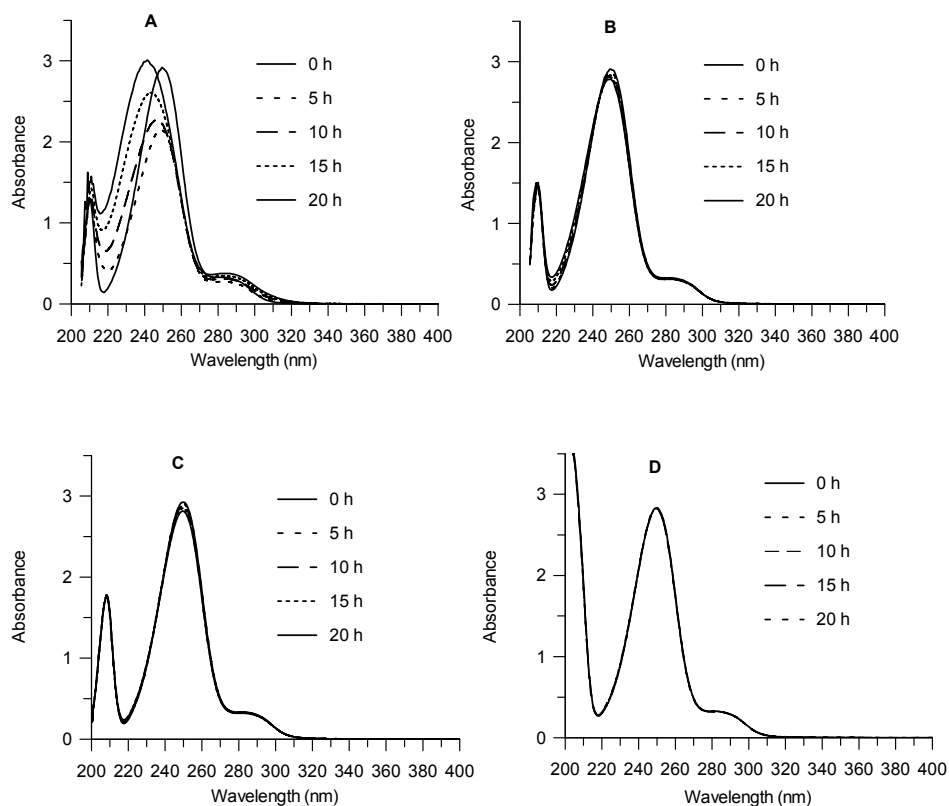


Figure 3. UV spectra recorded after incubation of **3** (50 mM) and **6** (2 mM) in 20 mM NaH_2PO_4 buffer at pH 7.3 with A) 4-OT F50A mutant, B) WT 4-OT, C) 4-OT P1A mutant, D) no enzyme (control sample).

not show significant absorbance above 220 nm.^[44] During the experiment with WT 4-OT and the 4-OT P1A mutant a negligible decrease at 250 nm was observed (Figures 3B and 3C). We did not detect any alteration in the UV spectra of the control sample (i.e., reaction mixture without enzyme) (Figure 3D).

^1H NMR experiments were performed to detect the products and clarify the reactions taking place in the presence of WT 4-OT and the 4-OT F50A mutant (Figure S1). In separate experiments, WT 4-OT, 4-OT F50A, and 4-OT P1A (2.0 mol% compared to **6**) were incubated with **3** (30 mM) and **6** (15 mM) in NaD_2PO_4 buffer (20 mM, pD 7.6). First we observed that during all experiments, equilibrium between hydrated (63%) and unhydrated (37%) form of **3** was reached (≤ 5 min) in the same manner as we described for the self-condensation of **3** while the H-D exchange of the acidic protons of **3** was again observed in the reaction mixtures incubated with WT 4-OT and 4-OT F50A.

Subsequently, we calculated the yield^[41] of products **7** and **8** in course of time for each reaction mixture. Analysis of the ^1H NMR spectra of the 4-OT F50A-catalyzed reaction revealed accumulation of aldol coupling product **7** to ~36% after 1 d, after which it

decreased to 20% after 14 d (Figure S2). In addition, the data showed that the yield of **8** steadily increased in time to 15% after 14 d. These observations suggest a relatively fast aldol addition of **3** to **6** to yield **7** and a relatively slow subsequent dehydration of **7** to **8**, which results in initial accumulation of **7**. Characteristic spectroscopic signals of **7** (at 5.23 and 9.74 ppm)^[45], the hydrate of **7** (i.e. **7'**, at 4.94 ppm. See Scheme S4 for structure of **7'**), and **8** (at 2.03 and 9.49 ppm)^[46,47] present in the reaction mixture incubated with 4-OT F50A are shown in Figure S1. No ¹H NMR signals corresponding to the hydrated form of **8** (conversion of the carbonyl moiety into diol) were detected throughout the entire course of the reaction. The ¹H NMR data also indicated activity of WT 4-OT for the cross-coupling of **3** and **6** albeit lower than that of the 4-OT F50A mutant (Figure S1). For the control experiment (i.e. the reaction mixture without enzyme) and the reaction mixture incubated with 4-OT P1A, only trace amounts of **7**, **7'**, and **8** were detected after 14 d.

In contrast to the UV experiments during which no absorbance corresponding to **8** was observed, the presence of signals for product **8** in the ¹H NMR spectra of the reaction mixture incubated by 4-OT F50A provided evidence that **7** was slowly converted to **8**. This anomaly may be explained by the different molar ratios of compounds **3** to **6** applied during the UV (**3**/**6** = 25:1) and ¹H NMR (**3**/**6** = 2:1) experiments and by the fact that the ¹H NMR signals corresponding to **8** were detected after 4 d (Figure S2) while the UV experiments were followed for a shorter time period of 20 h.

Compound **5** ($\lambda_{\text{max},5} = 234 \text{ nm}$) was detected, by ¹H NMR spectroscopy, in the reaction mixtures incubated with WT 4-OT and the 4-OT F50A mutant, which indeed explains the appearance of the absorbance at ~241 nm witnessed during the UV experiments (vide supra and Figure 3A). This observation means that self-condensation of **3** into **5** and cross-coupling of **3** and **6** take place simultaneously and are in competition with each other. In conclusion, the ¹H NMR data revealed that WT 4-OT and the 4-OT F50A mutant catalyze the cross-coupling of **3** and **6** and the Pro-1 residue is essential for catalysis of this reaction. Furthermore, the mutation of Phe-50 to Ala enhances the activity of 4-OT for cross-coupling of **3** and **6**. Whether the dehydration reaction (**7** to **8**) is enzyme-catalyzed or buffer-catalyzed requires further investigation.

Next, we examined whether 4-OT catalyzes the cross-coupling of **3** and **9**, a reaction which yields product **10** (Scheme 2). In separate experiments, WT 4-OT, 4-OT F50A, and 4-OT P1A (2.0 mol% compared to **9**) were incubated with **3** (30 mM) and **9** (15 mM) in NaD₂PO₄ buffer (20 mM, pD 7.6). During all experiments we noticed the same equilibrium between hydrated (63%) and unhydrated (37%) form of **3** as described during self-condensation of **3** (vide supra, Figure S3). Also, the H-D exchange of the acidic protons of **3** was observed in the reaction mixtures incubated with WT 4-OT and 4-OT F50A mutant (Figure S3). The protons of the methyl group of compound **9** are acidic and we noticed that the methyl signal of **9** was missing in the ¹H NMR spectra recorded of the reaction mixtures incubated with WT 4-OT and 4-OT F50A mutant for 1 h as a result of >99% H-D exchange. No H-D exchange of the acidic protons of **9** was observed in the control sample (without enzyme) after 1 h whereas only negligible H-D exchange was detected in the reaction mixture incubated with the 4-OT

P1A mutant after 1 h (7%). These observations clearly demonstrated that H-D exchange of the acidic protons of **9** is catalyzed by WT 4-OT and 4-OT F50A and indicated that the Pro-1 residue is essential for catalysis of the H-D exchange within **9**.

We analyzed the ^1H NMR data to obtain the yield^[41] of **10** in the reaction mixtures incubated with WT 4-OT and 4-OT F50A (Figures S3 and S4). The yield of **10** reached steady levels of 38 and 44% (based on substrate **9**) after 4 d in the reaction mixtures incubated with WT 4-OT and 4-OT F50A, respectively. Prolonged incubation of up to 8 d did not increase yields in both experiments. Signals corresponding to both diastereoisomers of **10** were detected in the ^1H NMR spectra of both the WT 4-OT and the 4-OT F50A sample (Figures S3 and S5). No signals corresponding to **10** were detected in the control sample (reaction mixture without enzyme) and the 4-OT P1A sample after 4 d. The ^1H NMR data indicated that WT 4-OT and 4-OT F50A both catalyze the cross-coupling of **3** and **9** and that the Pro-1 residue is crucial for catalysis (Scheme S5). Furthermore, when synthetic WT 4-OT was used instead of recombinant WT 4-OT, under otherwise identical reaction conditions, a similar yield of **10** was obtained (Figure S5). This excludes the possibility that catalysis was effected by a contaminating enzyme from the expression strain and confirms that 4-OT is responsible for catalysis.

In addition to those of **10**, signals corresponding to **5** were observed as well in the ^1H NMR spectra of the reaction mixture incubated with WT 4-OT and the 4-OT F50A mutant (Figure S3). The yield of **5** reached to 25 and ~30% (based on substrate **3**) after 4 and 8 d, respectively, in the sample containing the 4-OT F50A mutant. This observation reaffirmed that the 4-OT F50A mutant catalyzes the self-condensation of **3**. This means that the cross-coupling of **3** and **9** into **10** and the self-condensation of **3** into **5** are two competing conversions taking place simultaneously, having a reducing effect on the yield of **10**. No ^1H NMR signals corresponding to the dehydration product of **10**, nor the hydrated form of **10** (conversion of carbonyl moiety to diol), were detected during the entire course of the reaction. To the best of our knowledge, **10** has not been reported in the literature before. We have chemically synthesized **10** (Scheme S1) and found that its spectroscopic data (exact mass, ^1H and ^{13}C NMR spectroscopy) are identical to those of enzymatically obtained **10** (Figure S5).

Having established that WT 4-OT and 4-OT F50A catalyze various intermolecular aldolizations, we next investigated whether these enzymes can catalyze the intramolecular aldol-cyclizations of hexanedial (**11**) and heptanedial (**14**) (Scheme 2). Dials **11** and **14** were synthesized *in situ* from *trans*-1,2-cyclohexanediol (**17**) and *trans*-1,2-cycloheptanediol (**18**) based on a modified literature procedure (Scheme S6).^[48] First, the conversion of **11** to the cyclic aldehyde **12**, which may dehydrate to α,β -unsaturated aldehyde **13**, was monitored by UV spectroscopy. The sets of spectra recorded from the experiments with WT 4-OT and the 4-OT F50A mutant both show an increase of absorbance at 245 nm in course of time, which presumably corresponds to the formation of **13** ($\lambda_{\text{max},13} = 236$ nm, 99.5% EtOH) (Figure S6).^[49–51] To ascertain this finding, the enzymes WT 4-OT and 4-OT F50A (1.0 mol% compared to **11**) were incubated separately with **11** (15.4 mM in 20 mM

NaD_2PO_4 , $\text{pD} = 7.6$) and reaction progress was followed by ^1H NMR spectroscopy (Figure S7). A control reaction was also carried out using the same reaction mixture but without enzyme. We calculated the yield^[41] of compounds **12** and **13** in course of time from their characteristic ^1H NMR peaks (Figure S7). Since **11** and **12** are present in equilibrium with their corresponding hydrated form (monohydrate **11'**, dihydrate **11''** and hydrate **12'**), their presence is determined by the sum of the unhydrated and hydrated forms (i.e. presence of **11** = **11** + **11'** + **11''**). The hydrated form of **13** was not observed.

As illustrated in Figure S8, the yield of aldol coupling product **12** was ~67% in the reaction mixture incubated with 4-OT F50A after 24 h while the yield of dehydrated product **13** was ~23% after 24 h. These observations imply the accumulation of pharmaceutically important compound **12**^[50] in this period of time as a result of a slower dehydration step (i.e., **12** to **13**) compared to the aldol cyclization step (i.e., **11** to **12**). The yields of **12** and **13** were 40 and 10%, respectively, in the reaction mixture incubated with WT 4-OT after 24 h. The yields of **12** and **13** only reached to 10% and 1%, respectively, in the control sample without enzyme after 24 h. These findings confirm that both WT 4-OT and the 4-OT F50A mutant catalyze the intramolecular cyclization of **11**. It also indicates that a single site mutation in the active site of 4-OT, Phe-50 to Ala, significantly improves the aldol cyclization activity of 4-OT. Clarification on whether 4-OT WT and/or 4-OT F50A catalyze the dehydration step requires further investigation however.

The cyclization of **14** was monitored using the same methods and conditions as described for **11**. UV spectroscopic assays showed an increase of absorbance at 236 nm for reaction mixtures incubated with WT 4-OT and 4-OT F50A (Figure S10) within 12 h. Increase in the absorbance at 236 nm presumably corresponds to the formation of **16** ($\lambda_{\text{max},16} = 230 \text{ nm}$)^[52] via dehydration of **15**. Incubation of **14** in the absence of enzyme (control sample) or in the presence of 4-OT P1A resulted in a minor increase of absorbance at 236 nm, confirming that 4-OT and its Pro-1 residue are essential for catalysis. The ^1H NMR spectroscopic assays indicated that the yield of **15** was ~81% in the reaction mixture incubated with 4-OT F50A after 21 h [Figures S11 and S12, yield of **15** = **15** + **15'** (monohydrate)]. Only a small amount of compound **16** (11%) was observed after 21 h (Figures S11 and S12) indicating that the relatively fast aldol-cyclization step was followed by relatively slow dehydration (the hydrate of **16** was neither observed in the sample containing WT 4-OT nor in the presence of the 4-OT F50A mutant). The yields of **15** were ~42 and 6% in the WT 4-OT and control samples after 21 h, respectively. The yield of **16** reached to ~8% (21 h) in the reaction mixture containing WT 4-OT. A negligible amount of compound **16** (1.5%, 21 h) was detected in the control sample. In conclusion, these data clearly demonstrate that both WT 4-OT and the 4-OT F50A mutant catalyze the intramolecular cyclization of **14**. To clarify whether the dehydration reaction is enzyme-catalyzed or buffer-catalyzed requires further investigation. The results also showed that mutation of Phe-50 into Ala enhances the activity of 4-OT for intramolecular cyclization of **14**. Additionally, to eliminate the possible role of any contaminating enzyme from *E. coli* in catalysis of the intramolecular cyclization of **11** and **14**, we performed separate experiments in which recombinant and synthetic WT

4-OT were incubated, under identical conditions, with either compound **11** or **14**, and the reactions were monitored by UV spectroscopy. The increases of absorbance at 245 nm (in the presence of **11**) and 236 nm (in the presence of **14**) were similar for both recombinant and synthetic WT 4-OT (Figures S9 and S13). These experiments indeed confirmed that the 4-OT enzyme is responsible for catalysis of the intramolecular cyclization of **11** and **14**. Note that all of the four reaction products **12**, **13**, **15** and **16** are valuable building blocks in organic synthesis, especially for the synthesis of pharmaceuticals.^[53–61]

In summary, we have demonstrated that 4-OT promiscuously catalyzes different types of aldol reactions, namely intermolecular self-condensation, intermolecular cross-coupling and intramolecular cyclization. Furthermore, we have demonstrated fast H-D exchange of acidic protons of aldehyde **3** and ketone **9**. These H-D exchange reactions were confirmed to be enzyme-catalyzed and the essential catalytic role of Pro-1 was verified by site-directed mutagenesis. This unprecedented activity indicates that the active site of 4-OT can deprotonate carbonyl compounds, thereby providing additional evidence for a mechanism for the 4-OT catalyzed aldol reactions in which the substrate is activated for nucleophilic addition via Pro-1 dependent formation of an enolate or enamine intermediate. Finally, this study confirms that the substitution of only a single amino acid in the active site of 4-OT (F50A) enhances the promiscuous aldolase activities of 4-OT. These findings can be the beginning of a quest for novel 4-OT variants with strongly enhanced aldolase activity, but that lack dehydration activity, using enzyme engineering approaches.

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41. Here, and throughout the entire article, yield (%) of a compound at t_x is defined as the amount of moles of the compound (times 100) present in the reaction mixture at t_x as compared to the moles of starting material at t_0 : $\text{yield (\%)} = (N_{\text{product at } t = x} / N_{\text{starting material at } t = 0}) * 100$.
42. 4-OT was synthesized by GenScript USA Inc. (Piscataway, NY). For folding of chemically synthesized 4-OT into active homoheptamer see: M. C. Fitzgerald, I. Chernushevich, K. G. Standing, S. B. H. Kent, C. P. Whitman, *J. Am. Chem. Soc.* **1995**, *117*, 11075–11080.
43. Note that compound **3** is extremely volatile and therefore the evaporation of **3** may limit the conversion of **3** into **5**. We used 18-crown-6 (signal at 3.68 ppm) as internal standard to monitor the amount of evaporation of **3** which is necessary to calculate the yield of **5**.
44. Assumption based on the UV absorbance spectrum of 3-phenyl-3-hydroxy-propanal which is structurally akin to 3-hydroxy-2-methyl-3-phenylpropanal (**7**). See reference 28.
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Supporting information

Materials

The sources for the buffers, solvents, and components of Luria-Bertani (LB) media are reported elsewhere.^[1] High purity synthetic 4-OT was purchased from GenScript USA Inc. (Piscataway, NY) and folded into the active homohexamer as described before.^[2] Chemicals were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO). Compounds **11** and **14** were synthesized from *trans*-cyclohexane-1,2-diol and *trans*-cycloheptane-1,2-diol according to a modified literature procedure (Scheme S6).^[3]

General methods

Standard molecular biology techniques were performed based on methods described elsewhere.^[4] Protein analysis was performed by polyacrylamide gel electrophoresis (PAGE) using sodium dodecyl sulfate (SDS) gels containing polyacrylamide (10%). Coomassie brilliant blue was used to stain the gels. Protein concentrations were determined based on the Waddell method.^[5] Enzymatic assays were performed on a V-650 or V-660 spectrophotometer from Jasco (IJsselstein, The Netherlands). ¹H NMR spectra were recorded on a Varian Inova 500 (500 MHz) spectrometer using a pulse sequence for selective presaturation of the water signal. Chemical shifts for protons are reported in parts per million scale and are referenced to H₂O (4.80 ppm).

Expression and purification of WT 4-OT, 4-OT F50A and 4-OT P1A

The WT 4-OT, 4-OT F50A and 4-OT P1A enzymes were produced in *E. coli* BL21 (DE3) as native proteins (without His-tag) using the pET20b(+) expression system as described before.^[6] The construction of the expression vectors and the purification procedure for 4-OT and the mutant enzymes were reported previously.^[6] Before applying the 4-OT P1A mutant, which has essentially no aldolase activity, we first confirmed that purified 4-OT P1A was catalytically active by measuring its promiscuous oxaloacetate decarboxylase activity.^[7]

UV spectroscopic assay for self-condensation of propanal (3)

The self-condensation of propanal (**3**) was monitored by following the increase in absorbance at 234 nm which corresponds to the formation of 2-methyl-2-pentenal (**5**). The enzyme (150 μM) was incubated in a 1 mm cuvette with **3** (50 mM) in 20 mM NaH₂PO₄ buffer (pH 7.3; 0.3 mL final volume) and the reaction was followed for 20 h at room temperature. UV spectra were recorded from 200 to 400 nm.

Preparation of NaD₂PO₄ buffer

NaH₂PO₄ buffer (20 mL, 20 mM; pH 7.3) was lyophilized. Subsequently, the residue was dissolved in D₂O (2 mL), and stirred for 60 min. This solution was again lyophilized, dissolved in D₂O (2 mL), and stirred for 60 min. The resulting mixture was lyophilized once

more, after which the residue was dissolved in D₂O (20 mL) yielding a stock solution of NaD₂PO₄ (20 mM; pD 7.6).

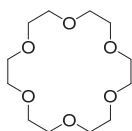
Redissolving 4-OT in NaD₂PO₄ buffer

A Vivaspin 2 concentrator (from Sartorius Stedim Goettingen, Germany) with a cut-off filter of 5000 Da was washed four times with H₂O by centrifugation (4000 rpm, 20 min). Subsequently, the concentrator was charged with a solution of 4-OT (either wild-type or mutant; 300 µL with concentration of ~10 mg/mL in 20 mM NaH₂PO₄ buffer, pH 7.3) and centrifuged (4000 rpm, 30 min). The enzyme was retained on the filter and redissolved in NaD₂PO₄ (200 µL, 20 mM; pD 7.6) and centrifuged (4000 rpm, 30 min). Once more, the remaining enzyme on the filter was redissolved in NaD₂PO₄ (300 µL, 20 mM; pD 7.6), after which the final enzyme concentration was determined.

¹H NMR spectroscopic assay for self-condensation of propanal (3)

In separate experiments, WT 4-OT, 4-OT F50A and 4-OT P1A (290 µM) were incubated with **3** (30 mM) and 18-crown-6 ether (internal standard; 2.15 mM) at room temperature in NaD₂PO₄ buffer (20 mM; pD 7.6, final volume of 650 µL in an NMR tube). A control sample was prepared containing all components except for the enzyme. ¹H NMR spectra were recorded ~1 h after incubation, and then after 1, 4, 8 and 14 d. ¹H NMR spectroscopic data (in 20 mM NaD₂PO₄ buffer, pD 7.6) of enzymatically obtained **5** are similar to those of an authentic standard of **5**. The hydrated form of **5** was not observed.

Internal standard 18-crown-6 (1,4,7,10,13,16-hexaoxacyclooctadecan)



¹H NMR (500 MHz, 20 mM NaD₂PO₄; pD 7.6): δ 3.68 (s, 24H)

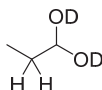
Propanal (**3**)



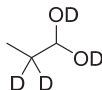
¹H NMR (500 MHz, 20 mM NaD₂PO₄; pD 7.6): δ 9.69 (t, *J* = 1.3 Hz, 1H), 2.57 (dq, *J* = 7.3, 1.3 Hz, 2H), 1.06 (t, *J* = 7.3 Hz, 3H)

Propanal-2,2- d_2 

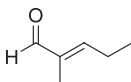
^1H NMR (500 MHz, 20 mM NaD_2PO_4 ; pD 7.6): δ 9.69 (s, 1H), 1.03 (s, 3H)

Propane-1,1-diol- d_2 (hydrate of **3**)

^1H NMR (500 MHz, 20 mM NaD_2PO_4 ; pD 7.6): δ 4.96 (t, J = 5.5 Hz, 1H), 1.59 (dq, J = 7.5, 5.5 Hz, 2H), 0.92 (t, J = 7.5 Hz, 3H)

Propane-2,2- d_2 -1,1-diol- d_2 (**3d'**-**d2**)

^1H NMR (500 MHz, 20 mM NaD_2PO_4 ; pD 7.6): δ 4.95 (s, 1H), 0.88 (s, 3H)

2-Methyl-2-pentenal (**5**)

^1H NMR (500 MHz, 20 mM NaD_2PO_4 ; pD 7.6): δ 9.29 (s, 1H), 6.81 (t, J = 7.6 Hz, 1H), 2.41 (dq, J = 7.6, 7.6 Hz, 2H), 1.71 (s, 3H), 1.09 (t, J = 7.6 Hz, 3H)

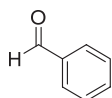
UV spectroscopic assay for cross-coupling of propanal (**3**) and benzaldehyde (**6**)

The cross-coupling of **3** and **6** was monitored by following the decrease in absorbance at 250 nm ($\lambda_{\text{max},6} = 250$ nm) indicating depletion of **6**. Simultaneously, the increase in absorbance at 288 nm, corresponding to the formation of **8** ($\lambda_{\text{max},8} = 288$ nm), was monitored. The enzyme (150 μM) was incubated in a 1 mm cuvette with **3** (50 mM) and **6** (2 mM) in 20 mM NaH_2PO_4 buffer (pH 7.3; 0.3 mL final volume) and the reaction was followed for 20 h at room temperature. UV spectra were recorded from 200 to 400 nm.

¹H NMR spectroscopic assay for cross-coupling of propanal (3) and benzaldehyde (6)

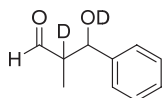
The enzymes WT 4-OT, 4-OT F50A and 4-OT P1A (290 μM), respectively, were incubated with **3** (30 mM), **6** (15 mM) and 18-crown-6 ether (internal standard; 2.15 mM) at room temperature in NaD₂PO₄ buffer (20 mM; pD 7.6, final volume of 650 μL in an NMR tube). A control sample was prepared with all the components except for the enzyme. ¹H NMR spectra were recorded ~2 h after incubation, and then after 1, 4, 8 and 14 d. ¹H NMR spectroscopic data of **7**^[8,9] are in accordance with data in the literature. ¹H NMR spectroscopic data (in 20 mM NaD₂PO₄ buffer, pD 7.6) of enzymatically prepared **8** are identical to those of an authentic standard of **8**. Hydrated forms of **6** and **8** were not observed.

Benzaldehyde (6)



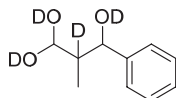
¹H NMR (500 MHz, 20 mM NaD₂PO₄; pD 7.6): δ 9.94 (s, 1H), 7.97 (d, *J* = 7.9 Hz, 2H), 7.76 (d, *J* = 7.5 Hz, 1H), 7.63 (dd, *J* = 7.9, 7.5 Hz, 2H)

3-(Hydroxy-*d*)-2-methyl-3-phenylpropanal-2-*d* (7)

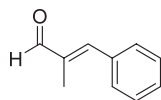


¹H NMR (500 MHz, 20 mM NaD₂PO₄; pD 7.6): δ 9.74 (s, 1H), 7.48 – 7.36 (m, 5H), 5.23 (s, 1H), 1.03 (s, 3H)

2-Methyl-3-phenylpropane-2-*d*-1,1,3-triol-*d*₃ (7')



¹H NMR (500 MHz, 20 mM NaD₂PO₄; pD 7.6): δ 7.48 – 7.36 (m, 5H), 5.21 (s, 1H), 4.94 (s, 1H), 0.94 (s, 3H)

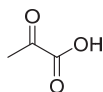
(E)-2-Methyl-3-phenylacrylaldehyde (**8**)

^1H NMR (500 MHz, 20 mM NaD_2PO_4 ; pD 7.6): δ 9.49 (s, 1H), 7.67 (d, $J = 7.5$ Hz, 2H), 7.55 – 7.50 (m, 4H), 2.03 (s, 3H)

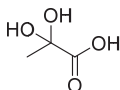
 ^1H NMR spectroscopic assay for cross-coupling of propanal (**3**) and pyruvate (**9**)

In separate experiments, the enzymes WT 4-OT, 4-OT F50A and 4-OT P1A (290 μM) were incubated with **3** (30 mM) and **9** (15 mM) and 18-crown-6 ether (internal standard; 2.15 mM) at room temperature in NaD_2PO_4 buffer (20 mM; pD 7.6, final volume of 650 μL in an NMR tube). The control sample was prepared with all the components except for the enzyme. ^1H NMR spectra were recorded ~ 1 h after incubation, and then after 1, 4, 8 and 14 d. The yield of product **10** was determined on the basis of the sum of the integrations of the two aldehyde signals of the two diastereoisomers of **10**. All other proton signals of **10** were either invisible as a result of H-D exchange or overlap with signals of starting materials **3** and **9**. To the best of our knowledge, synthesis of 2-hydroxy-2,3-dimethyl-4-oxobutanoic acid (**10**) has not been reported in the literature so far. Therefore, we have chemically synthesized **10** to confirm the identity of enzymatically obtained **10**, and for full characterization with ^1H NMR, ^{13}C NMR, and exact mass spectroscopy (vide infra).

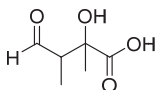
The experiment with WT 4-OT was repeated in NaH_2PO_4 buffer (20 mM; pH 7.3) with D_2O (10% v/v) to avoid H-D exchange and enable the detection of all proton signals of **10** by ^1H NMR spectroscopy (Figure S3). Propanal (**3**, 50 mM) and pyruvate (**9**, 50 mM) were incubated with WT 4-OT (90 μM) in NaH_2PO_4 buffer (20 mM; pH 7.6, final volume of 650 μL in an NMR tube). Reaction progress was monitored with ^1H NMR spectroscopy which revealed formation of product **10**, as a result of cross-coupling of **3** and **9**, and of product **5** as a result of self-coupling of **3**. In contrast to the experiment in 100% NaD_2PO_4 buffer (vide supra), the hydrated form of **10** (i.e. **10'**) was observed in small quantities (<5% compared to **10**). This experiment was repeated in the absence of enzyme and in the presence of synthetic 4-OT WT^[2] instead of recombinant 4-OT. In the absence of enzyme, formation of product **10** was not observed while only trace amounts of **5** were detected. In the presence of synthetic 4-OT WT, formation of products **10** and **5** was observed in equal quantities as in the presence of recombinant 4-OT WT.

Pyruvate (**9**)

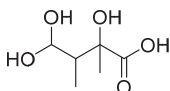
^1H NMR (500 MHz, 20 mM NaH_2PO_4 ; pH 7.3): δ 2.39 (s, 3H)

2,2-Dihydroxypropanoic acid (**9'**: hydrated **9**)

^1H NMR (500 MHz, 20 mM NaH_2PO_4 ; pH 7.3): δ 1.50 (s, 3H)

2-Hydroxy-2,3-dimethyl-4-oxobutanoic acid (**10**, enzymatically prepared)

^1H NMR (500 MHz, 20 mM NaH_2PO_4 ; pH 7.3): (diastereomer I) δ 9.74 (d, $J = 2.7$ Hz, 1H), 2.74 (dq, $J = 7.0, 2.7$ Hz, 1H), 1.44 (s, 3H), 1.08 (d, $J = 7.0$ Hz, 3H); (diastereomer II) δ 9.62 (d, $J = 0.9$ Hz, 1H), 2.74 (dq, $J = 7.0, 0.9$ Hz, 1H), 1.47 (s, 3H), 1.02 (d, $J = 7.0$ Hz, 3H)

2,4,4-Trihydroxy-2,3-dimethylbutanoic acid (**10'**)

The ^1H NMR spectroscopic data for **10'** are listed below (see chemically prepared **10**).

Chemical synthesis of 2-hydroxy-2,3-dimethyl-4-oxobutanoic acid (**10**)

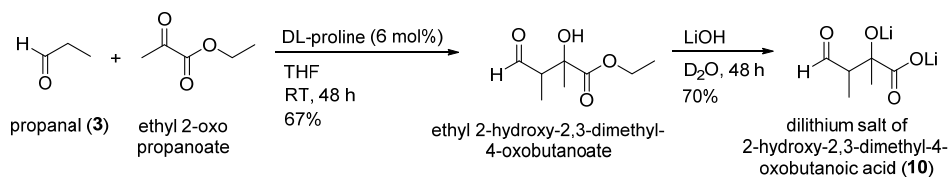
Compound **10** was synthesized by a two-step procedure. During the first step, an aldol coupling of propanal (**3**) and ethyl 2-oxopropanoate (commercially available), following a modified literature procedure,^[10] gave ethyl 2-hydroxy-2,3-dimethyl-4-oxobutanoate in 67% yield after column chromatography. Subsequently, ethyl 2-hydroxy-2,3-dimethyl-4-oxobutanoate was hydrolyzed with LiOH in D_2O to give 2-hydroxy-2,3-dimethyl-4-oxobutanoic acid (**10**). The ^1H NMR data of enzymatically obtained **10** matched those of chemically obtained **10** (Figure S5).

Ethyl 2-hydroxy-2,3-dimethyl-4-oxobutanoate

Ethyl 2-oxopropanoate (2.0 g, 17.2 mmol) and propanal (**3**, 4.0 g, 34.4 mmol) were dissolved in THF (25 mL). DL-proline (115 mg, 1.0 mmol) was added and the mixture was stirred for 48 h at room temperature. Reaction progress was monitored by thin layer chromatography (silica gel, hexanes/ethyl acetate 3/1). The solvent was evaporated *in vacuo* and the residue was submitted to column chromatography (silica gel, hexanes/ethyl acetate 5/1) to yield two diastereoisomers of ethyl 2-hydroxy-2,3-dimethyl-4-oxobutanoate (2.0 g, 11.5 mmol, 67%) in a ~55/45 ratio as a colorless oil. ¹H NMR (500 MHz, CDCl₃, 20°C); major diastereomer: δ 9.63 (s, 1H), 4.31 – 4.20 (m, 2H), 3.25 (b, 1H), 2.62 (q, *J* = 7.3 Hz, 1H), 1.43 (s, 3H), 1.28 (t, *J* = 7.2 Hz, 3H), 1.23 (d, *J* = 7.3 Hz, 3H); minor diastereomer: δ 9.74 (d, *J* = 2.9 Hz, 1H), 4.31 – 4.20 (m, 2H), 3.25 (b, 1H), 2.62 (dq, *J* = 7.1, 2.9 Hz, 1H), 1.50 (s, 3H), 1.30 (t, *J* = 7.1 Hz, 3H), 1.10 (d, *J* = 7.1 Hz, 3H); ¹³C NMR (125 MHz, CDCl₃, 20°C); major diastereomer: δ 202.72, 175.86, 74.15, 62.14, 53.13, 24.08, 13.96, 7.96; minor diastereomer: δ 203.16, 175.16, 75.03, 62.30, 52.81, 24.53, 14.00, 9.51; HRMS (ESI): *m/z* = 175.09665 [M+H]⁺ (calcd. 175.09650 for C₈H₁₅O₄).

2-Hydroxy-2,3-dimethyl-4-oxobutanoic acid (**10**).

A mixture of ethyl 2-hydroxy-2,3-dimethyl-4-oxobutanoate (112 mg, 0.64 mmol), LiOH (15.3 mg, 0.64 mmol) and D₂O (1.5 mL) was stirred for 2 d at room temperature. A ¹H NMR spectrum of an aliquot of the reaction mixture, diluted with D₂O, revealed ~70% conversion of ethyl 2-hydroxy-2,3-dimethyl-4-oxobutanoate into 2-hydroxy-2,3-dimethyl-4-oxobutanoic acid (**10**). The reaction mixture was washed with EtOAc (7 × 1.5 mL) to remove unhydrolyzed ester and ethanol. The D₂O layer was concentrated to ~1.0 mL *in vacuo* to remove residual EtOAc. The remaining D₂O layer was analyzed by ¹H NMR spectroscopy revealing the presence of two diastereomers of 2-hydroxy-2,3-dimethyl-4-oxobutanoic acid (**10**) in a ~1/1 ratio. The hydrated forms of both diastereomers (i.e. 2,4,4-trihydroxy-2,3-dimethylbutanoic acid **10'**) were also observed (~7 mol% relative to aldehyde **10**). ¹H NMR (500 MHz, D₂O, 20°C): (diastereomer I) δ 9.80 (d, *J* = 2.2 Hz, 1H), 2.78 (dq, *J* = 7.0, 2.2 Hz, 1H), 1.48 (s, 3H), 1.12 (d, *J* = 7.0 Hz, 3H); (diastereomer II) δ 9.67 (s, 1H), 2.78 (q, *J* = 7.0, 1H), 1.51 (s, 3H), 1.06 (d, *J* = 7.0 Hz, 3H); diastereomers of hydrate: δ 5.30 (d, *J* = 4.0 Hz, 1H) and 5.13 (d, *J* = 3.4 Hz, 1H), 2.12 – 2.08 (m, 1H) and 2.07 – 2.03 (m, 1H), 1.42 (s, 3H) and 1.34 (s, 3H), 1.02 (d, *J* = 7.1 Hz, 3H) and 0.94 (d, *J* = 7.1 Hz, 3H); ¹³C NMR (125 MHz, D₂O, 20°C): (two diastereomers) δ



Scheme S1. Chemical synthesis of **10**.

207.49 and 207.46, 181.22 and 180.76, 76.88 and 76.53, 53.78 and 52.78, 24.21 and 23.68, 8.72 and 7.47; HRMS (ESI): m/z = 159.08167 $[M+H]^+$ (calcd. 159.08154 for $C_6H_9O_4Li_2$).

Chemical synthesis of hexanedial (11) and heptanedial (14)

Dials **11** and **14** were synthesized *in situ* from *trans*-cyclohexane-1,2-diol (**17**) and *trans*-cycloheptane-1,2-diol (**18**) according to a modified literature procedure (Scheme S6).^[3] General procedure: a 100 mM solution of diol and a 100 mM solution of sodium periodate were prepared in D_2O . Each solution (500 μL) was transferred to a glass vial and the solution was mixed gently. After 1 h, a 1H NMR spectrum was recorded which showed quantitative conversion into the dial, which was in equilibrium with its mono- and dihydrates (**11'**, **11''**, **14'**, and **14''**), without any visible impurities. 1H NMR data of **11**^[3] and **14**^[11] are in accordance with data reported in the literature.

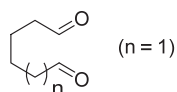
UV spectroscopic assay for cyclization of 11 and 14

In separate experiments, the enzymes WT 4-OT, 4-OT F50A and 4-OT P1A (150 μM) were incubated with dial (**11** or **14**, 16.7 mM) in a 1 mm cuvette at room temperature in NaH_2PO_4 buffer (20 mM, pH 7.3; 0.3 mL final volume). UV spectra were recorded from 200 to 400 nm at t = 6 and 12 h ($\lambda_{max,13}$ = 245 nm (Lit : 236 nm in 99.5% EtOH)^[12], $\lambda_{max,16}$ = 236 nm (Lit : 230 nm in 100% EtOH)^[13]). Control experiments (all components but without enzyme) were performed as well.

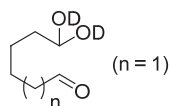
1H NMR spectroscopic assay for cyclization of 11 and 14

The enzymes WT 4-OT and 4-OT F50A (148 μM) were incubated separately with dialdehyde (**11** or **14**, 15.4 mM in 20 mM NaD_2PO_4 , pD = 7.6). Total volume of each reaction mixture was 650 μL in an NMR tube. The tubes were stored at room temperature, protected from light. 1H NMR spectra were recorded after 0, 2, 21, 27, 45, 50, 68, 75, 144, 216, 242, 333, 408, 503, 528 and 672 h. After 4 weeks, the enzymes were still fully active as determined by an enzymatic assay monitoring the enol-keto tautomerization of phenylpyruvate^[14] (5 mM phenylpyruvate, 1 $\mu g/mL$ 4-OT wildtype or 0.1 $\mu g/mL$ 4-OT F50A in NaH_2PO_4 buffer, pH 7.3). 1H NMR spectroscopic data of **13**^[15] and **16**^[16] are consistent with data in the literature. Hydrated forms of **13** and **16** were not observed.

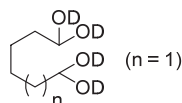
Hexanedial (**11**)



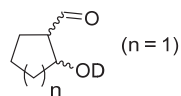
1H NMR (500 MHz, 20 mM NaD_2PO_4 ; pD 7.6): δ 9.71 (t, J = 2.1 Hz, 2H), 2.62 – 2.56 (m, 4H), 1.72 – 1.61 (m, 4H)

6,6-Di(hydroxy-*d*)hexanal (**11'**)

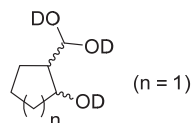
^1H NMR (500 MHz, 20 mM NaD_2PO_4 ; pD 7.6): δ 9.71 (t, $J = 2.1$ Hz, 1H), 5.06 (t, $J = 5.6$ Hz, 1H), 2.62 – 2.56 (m, 2H), 1.72 – 1.61 (m, 4H), 1.47 – 1.38 (m, 2H)

Hexane-1,1,6,6-tetraol- d_4 (**11''**)

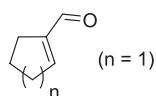
^1H NMR (500 MHz, 20 mM NaD_2PO_4 ; pD 7.6): δ 5.06 (t, $J = 5.6$ Hz, 2H), 1.72 – 1.61 (m, 4H), 1.47 – 1.38 (m, 4H)

2-(Hydroxy-*d*)cyclopentane-1-carbaldehyde (**12**)

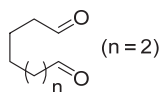
^1H NMR (500 MHz, 20 mM NaD_2PO_4 ; pD 7.6): (major diastereomer) δ 9.67 (s, 1H), 4.30 (m, 1H), 4.14 (m, 1H, overlapping with signal of compound 12' (hydrate)), 1.96 – 1.46 (m, 6H); characteristic signal of minor diastereomer: δ 9.81 (s, 1H)

(2-(Hydroxy-*d*)cyclopentyl)methanediol- d_2 (**12'**)

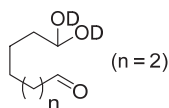
^1H NMR (500 MHz, 20 mM NaD_2PO_4 ; pD 7.6): (major diastereomer) δ 4.97 (s, 1H), 4.52 (m, 1H), 4.14 (m, 1H, overlapping with signal of compound 12), 1.96 – 1.46 (m, 6H); characteristic signal of minor diastereomer: δ 5.03 (s, 1H)

Cyclopent-1-ene-1-carbaldehyde (**13**)

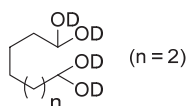
^1H NMR (500 MHz, 20 mM NaD_2PO_4 ; pD 7.6): δ 9.68 (s, 1H), 7.25 (s, 1H), 2.66 (t, J = 7.6 Hz, 2H), 2.49 (t, J = 7.0 Hz, 2H), 2.05 – 1.99 (m, 2H)

Heptanedial (**14**)

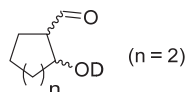
^1H NMR (500 MHz, 20 mM NaD_2PO_4 ; pD 7.6): δ 9.71 (t, J = 1.9 Hz, 2H), 2.59 – 2.54 (m, 4H), 1.69 – 1.61 (m, 4H), 1.44 – 1.35 (m, 2H)

7,7-di(hydroxy-*d*)heptanal (**14'**)

^1H NMR (500 MHz, 20 mM NaD_2PO_4 ; pD 7.6): δ 9.71 (t, J = 1.9 Hz, 1H), 5.05 (t, J = 5.6 Hz, 1H), 2.59 – 2.54 (m, 2H), 1.69 – 1.61 (m, 4H), 1.44 – 1.35 (m, 4H)

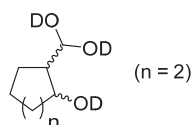
Heptane-1,1,7,7-tetraol-*d*₄ (**14''**)

^1H NMR (500 MHz, 20 mM NaD_2PO_4 ; pD 7.6): δ 5.05 (t, J = 5.6 Hz, 2H), 1.69 – 1.61 (m, 4H), 1.44 – 1.35 (m, 6H)

2-(Hydroxy-*d*)cyclohexane-1-carbaldehyde (**15**)

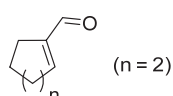
^1H NMR (500 MHz, 20 mM NaD_2PO_4 ; pD 7.6): (major diastereomer) δ 9.67 (s, 1H), 3.95 – 3.89 (m, 1H), 2.06 – 1.13 (m, 8H), signal of 1 proton overlaps with water signal at 4.80 ppm; characteristic signal of minor diastereomer: δ 9.72 (s, 1H)

(2-(hydroxy-*d*)cyclohexyl)methanediol-*d*₂ (**15'**)

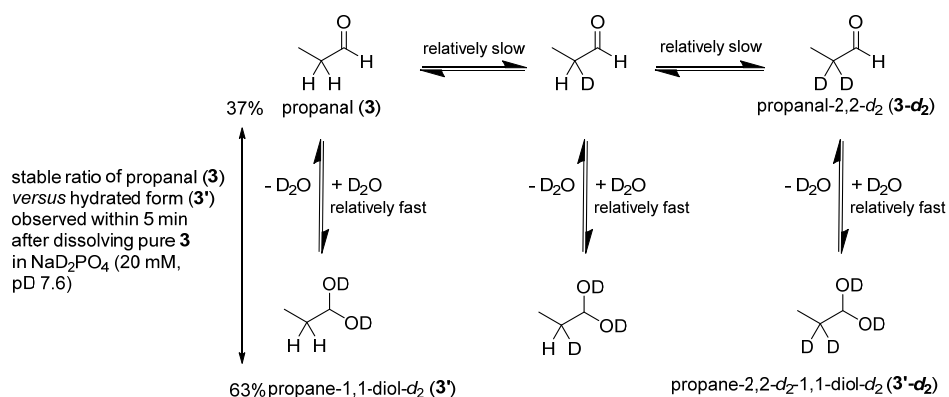


¹H NMR (500 MHz, 20 mM NaD₂PO₄; pD 7.6): (major diastereomer) δ 5.22 (s, 1H), 4.52 – 4.48 (m, 1H), 3.63 – 3.57 (m, 1H), 2.06 – 1.13 (m, 8H); minor diastereomer was not observed.

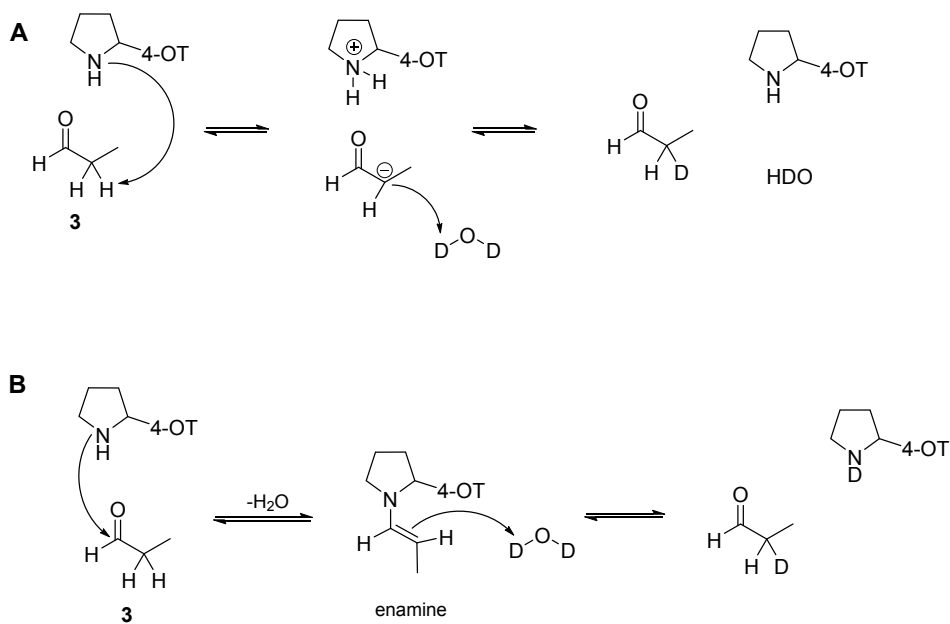
Cyclohex-1-ene-1-carbaldehyde (**16**)



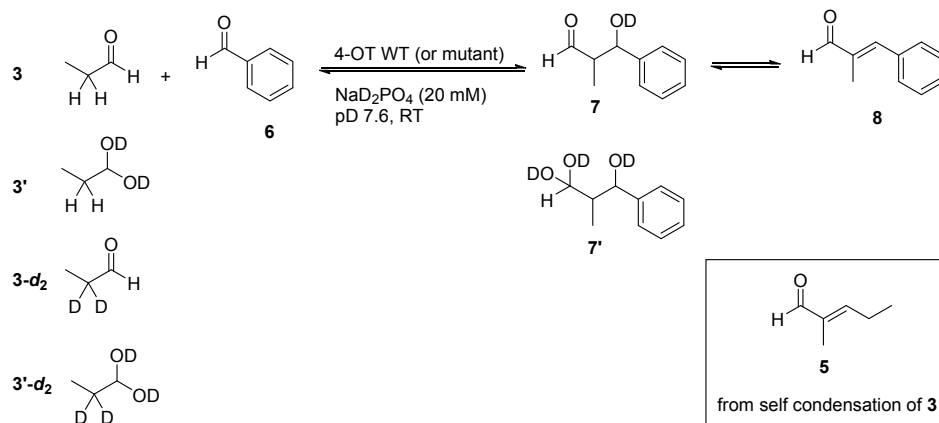
¹H NMR (500 MHz, 20 mM NaD₂PO₄; pD 7.6): δ 9.29 (s, 1H), 7.13 (s, 1H), 2.46 – 2.33 (m, 2H), 2.21 – 2.09 (m, 2H), 1.72 – 1.61 (m, 4H)



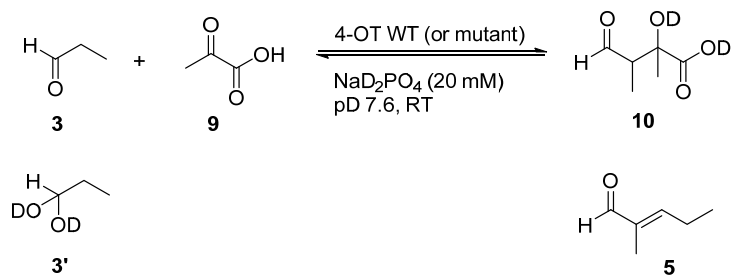
Scheme S2. Stable equilibrium between the hydrated (63%) and unhydrated (37%) form of **3** witnessed immediately after preparing sample (≤ 5 min, ¹H NMR spectroscopy) in the absence as well as presence of enzyme (4-OT WT, F50A, P1A). Hydrogen-deuterium exchange of the acidic protons of substrate **3** (monitored by ¹H NMR spectroscopy. See paragraph '¹H NMR spectroscopic assay for self-condensation of propanal (**3**)'). The exchange most likely only takes place at C2 of the unhydrated form of **3** (i.e. propanal) and not at C2 of the hydrated form (**3'**) since protons at C2 of the latter are not acidic. The rates for reaching equilibrium between unhydrated and hydrated form are relatively high compared to the rates of H-D exchange.



Scheme S3. Proposed mechanisms for the 4-OT-catalyzed hydrogen-deuterium exchange within propanal (**3**) and the role of Pro-1 as base (A) or as nucleophile (B).

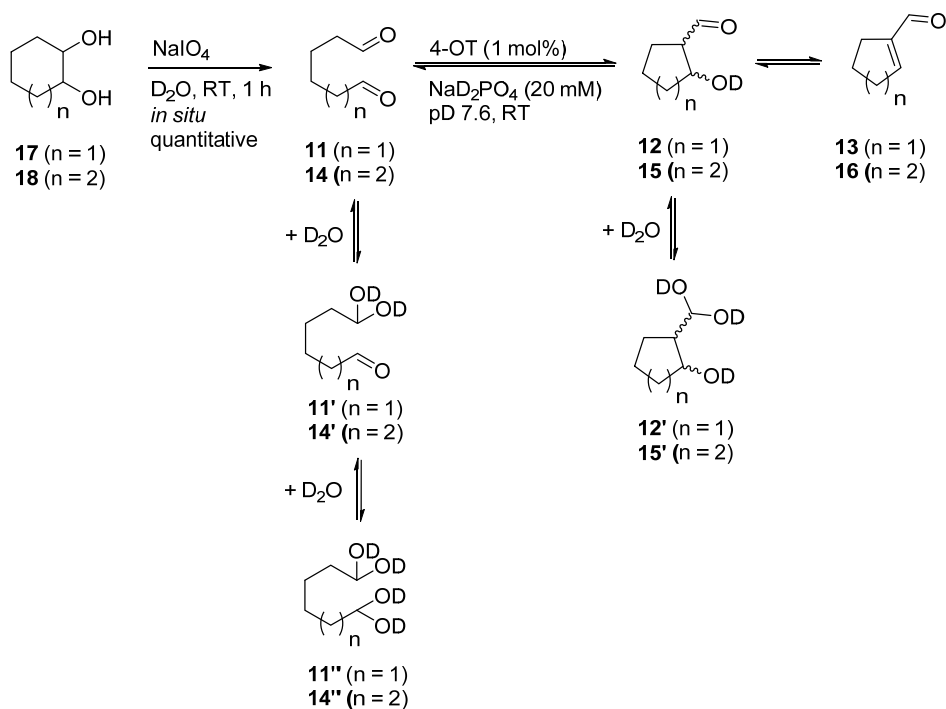


Scheme S4. Incubation of **3** and **6** in 20 mM NaD_2PO_4 buffer (pD 7.6) with 4-OT WT, 4-OT F50A, or 4-OT P1A.



Scheme S5. Incubation of 3 and 9 in 20 mM NaD_2PO_4 buffer (pD 7.6) with 4-OT WT, 4-OT F50A, 4-OT P1A, or synthetic 4-OT. Product 10 is not formed in the absence of enzyme.

6



Scheme S6. *In situ* preparation of hexanedial (11) and heptanedial (14), and their mono- and dihydrates (11', 11'', 14', and 14''), subsequent 4-OT-catalyzed cyclization into products 12 and 15, and final dehydration into α,β -unsaturated adducts 13 and 16. Hydrated forms of 13 and 16 were not observed.

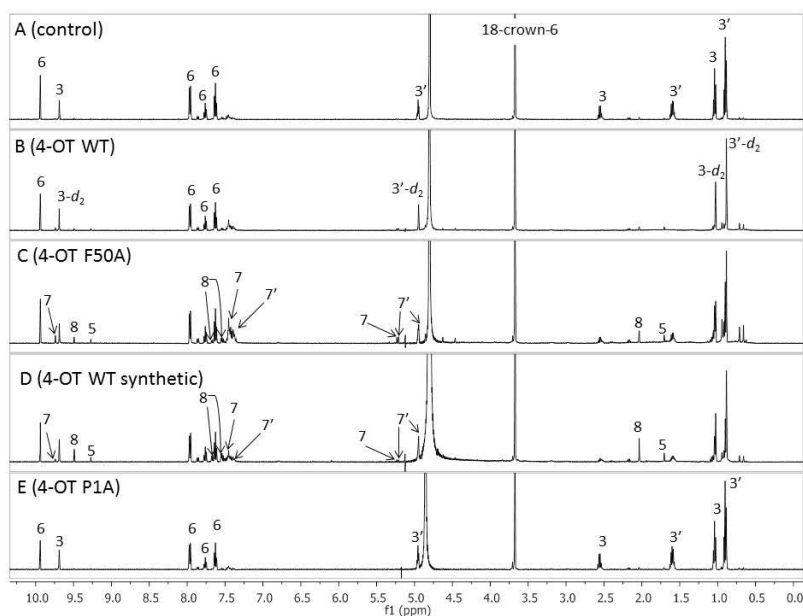


Figure S1. Stack plot of ^1H NMR spectra recorded after 4 d of incubation of **3** and **6** in 20 mM NaD_2PO_4 buffer (pD 7.6) with A) no enzyme; B) 4-OT WT; C) 4-OT F50A; D) 4-OT WT synthetic; E) 4-OT P1A (See Scheme S4 for reaction scheme). Spectrum A shows equilibrium was reached between unhydrated (i.e. **3'**) and hydrated forms of **3** and shows no formation of products **7**, **7'**, **8** nor **5** (as a result of self-condensation of **3**). Spectrum B shows that protons at C2 of **3** and **3'** have exchanged with deuterium resulting in formation of **3-d₂** and **3'-d₂** (see Scheme S2 for mechanism). Spectrum B furthermore shows little formation of products **7**, **7'**, **8** and **5** (indicative signals are specified in spectrum C). Spectra C and D show formation of products **7**, **7'**, **8** and **5** (only indicative signals are given). Signals of **3** and **6** not marked for the sake of clarity). Hydrated **8** was not observed. Spectrum E shows no formation of products **7**, **7'**, **8** and **5**, nor proton-deuterium exchange of **3**.

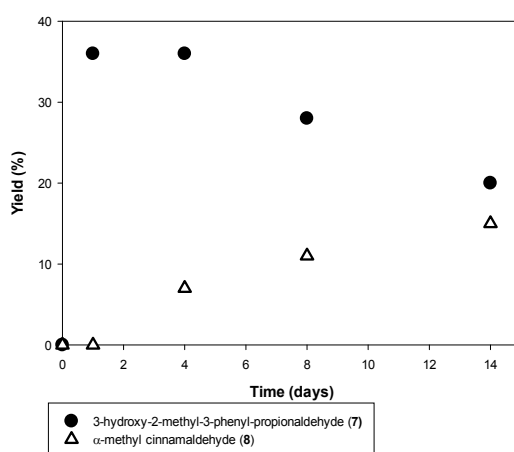


Figure S2. Yields (%) of **7** (●) and **8** (Δ) (based on **6**) in course of time in the reaction mixture (**3** + **6**) incubated with 4-OT F50A.

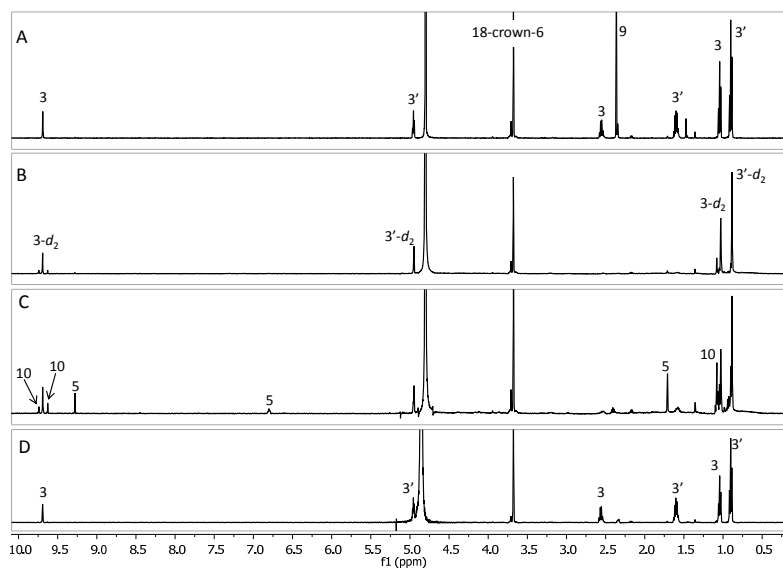


Figure S3. Stack plot of ^1H NMR spectra recorded after 4 d of incubation of **3** and **9** in 20 mM NaD_2PO_4 buffer (pD 7.6) with A) no enzyme; B) 4-OT WT; C) 4-OT F50A; D) 4-OT P1A (see Scheme S5 for reaction scheme). Spectrum A shows equilibrium was reached between unhydrated and hydrated (i.e. $3'$) forms of **3** and shows no formation of product **10** nor **5** (as a result of self-condensation of **3**). Spectrum B shows that protons at C2 of **3** and $3'$ and methyl protons of **9** have exchanged with deuterium resulting in formation of $3\text{-}d_2$, $3'\text{-}d_2$ (see Scheme S2 for mechanism), and $9\text{-}d_3$ (latter not visible in spectrum). Spectrum B furthermore shows little formation of product **10** (two diastereomers) and of **5** (indicative signals are specified in spectrum C). Spectrum C shows formation of **10** (two diastereomers) and **5** (only indicative signals are given). Signals of **3** are not marked for the sake of clarity). Spectrum D shows no formation of products **10** and **5**, nor proton-deuterium exchange of **3**. It does however indicate complete H-D exchange of acidic methyl protons of **9**.

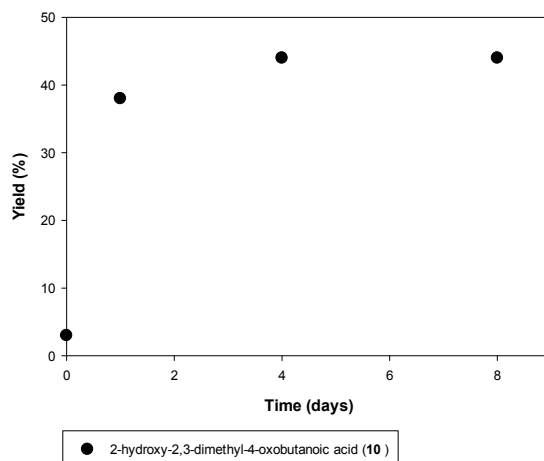


Figure S4. Yields (%) of **10** (based on **9**) in course of time in the reaction mixture (**3** + **9**) incubated with the 4-OT F50A mutant.

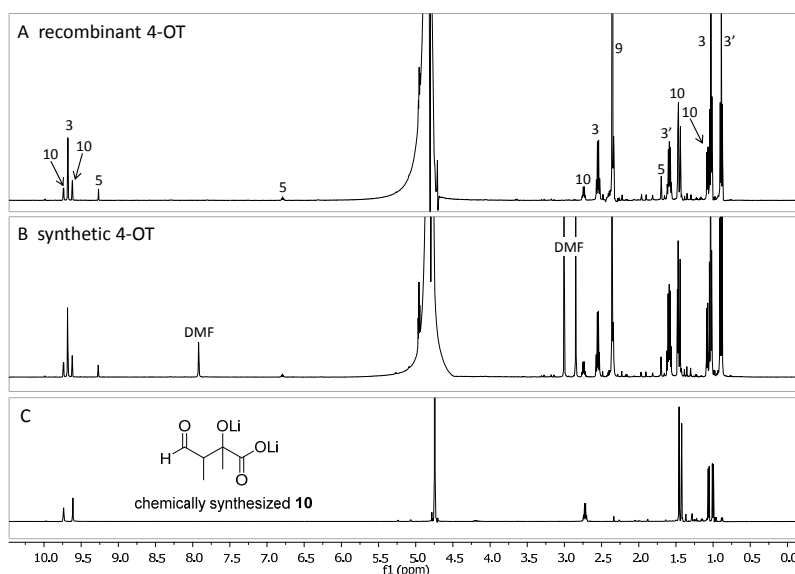


Figure S5. Stack plot of ^1H NMR spectra recorded after 4 d of incubation of **3** and **9** in 20 mM NaH_2PO_4 buffer (pH 7.3) with A) recombinant 4-OT WT and B) synthetic 4-OT WT (DMF used as internal standard). Spectrum C) shows the signals for chemically synthesized **10**. See Scheme S5 for reaction scheme.

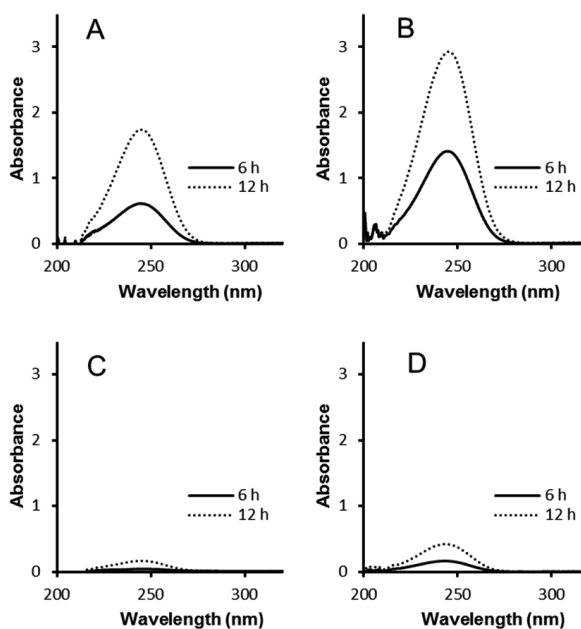


Figure S6. UV spectra recorded after incubation of **11** (16.7 mM) in 20 mM NaH_2PO_4 buffer at pH 7.3 with A) WT 4-OT, B) 4-OT F50A mutant, C) no enzyme (control sample) and D) 4-OT P1A mutant ($t = 6$ and 12 h). Increase of absorbance at 245 nm indicates formation of **13**.

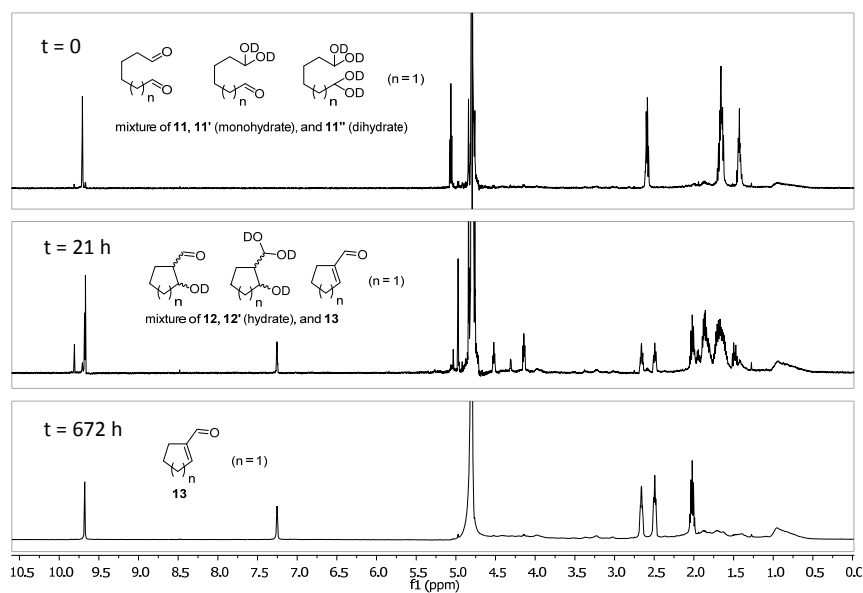


Figure S7. Cyclization of hexanedial **11** (15.4 mM) into **13** in NaD_2PO_4 (20 mM, pD = 7.6), catalyzed by 4-OT F50A (148 μM), monitored by ^1H NMR spectroscopy ($t = 0, 21, 672$ h) (See Scheme S6 for reaction scheme). The enzyme 4-OT is responsible for broad absorptions between ~ 2 and 0.5 ppm, especially well-visible in the bottom spectrum.

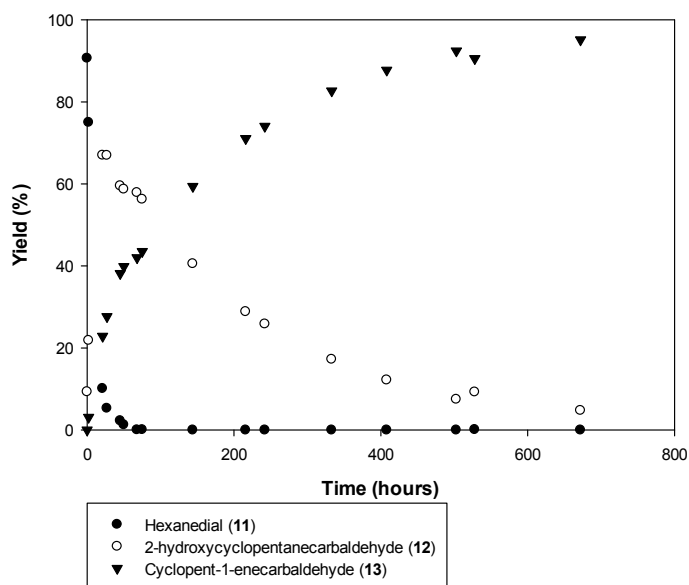


Figure S8. Plot of the presence (%) of **11** and yields (%) of **12** and **13** in course of time in the reaction mixture incubated with the 4-OT F50A mutant (in 20 mM NaD_2PO_4 buffer at pD 7.6).

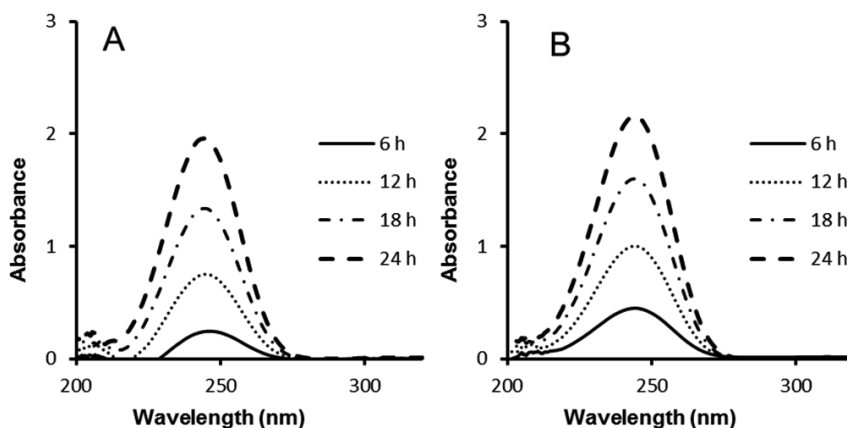


Figure S9. UV spectra recorded after incubation of **11** (16.7 mM) in 20 mM NaH_2PO_4 buffer at pH 7.3 with 0.075 mM A) recombinant WT 4-OT and B) synthetic WT 4-OT ($t = 6, 12, 18$ and 24 h).

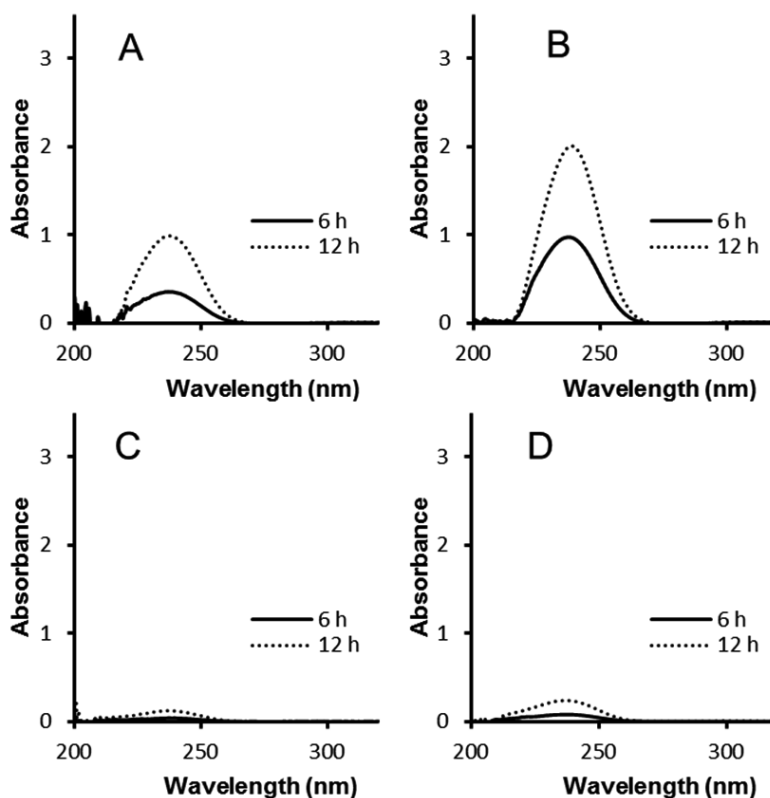


Figure S10. UV spectra recorded after incubation of **14** (16.7 mM) in 20 mM NaH_2PO_4 buffer at pH 7.3 with A) WT 4-OT, B) 4-OT F50A mutant, C) no enzyme (control sample) and D) 4-OT P1A mutant ($t = 6$ and 12 h). Increase of absorbance at 236 nm indicates formation of **16**.

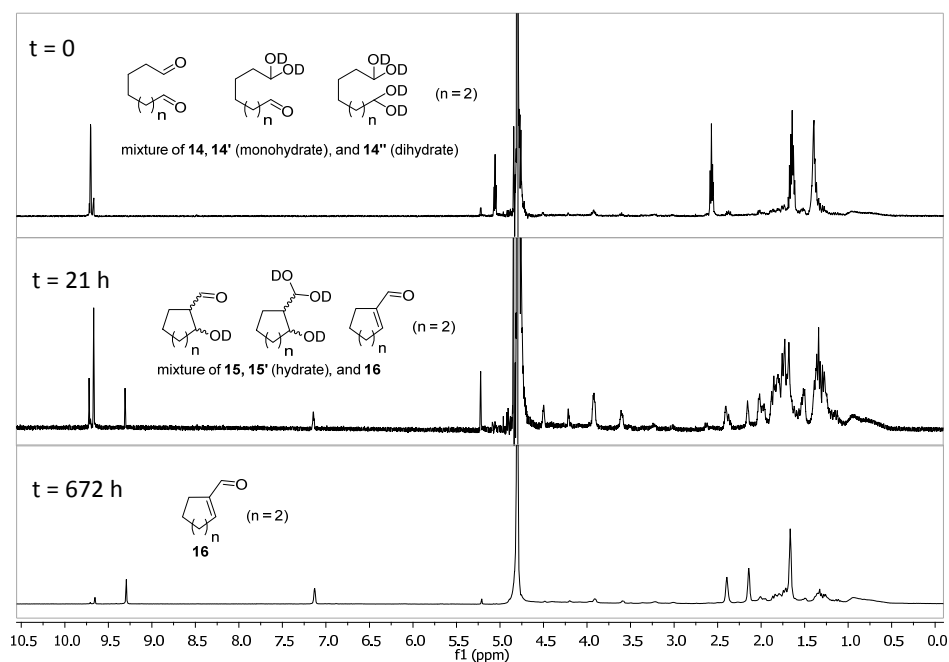


Figure S11. Cyclization of heptanedial **14** (15.4 mM) into **16** in NaD_2PO_4 (20 mM, pD = 7.6), catalyzed by 4-OT F50A (148 μM), monitored by ^1H NMR spectroscopy ($t = 0, 21, 672$ h) (See Scheme S6 for reaction scheme). The enzyme 4-OT is responsible for broad absorptions between ~ 2 and 0.5 ppm, especially well-visible in the bottom spectrum.

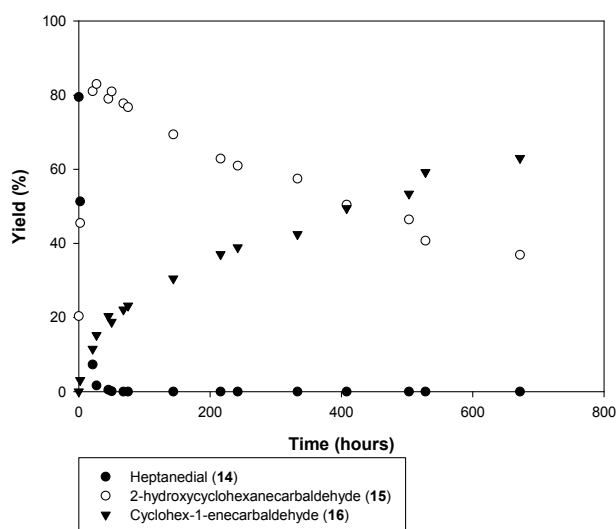


Figure S12. Plot of the presence (%) of **14** and yields (%) of **15** and **16** in course of time in the reaction mixture incubated with the 4-OT F50A mutant (in 20 mM NaD_2PO_4 buffer at pD 7.6).

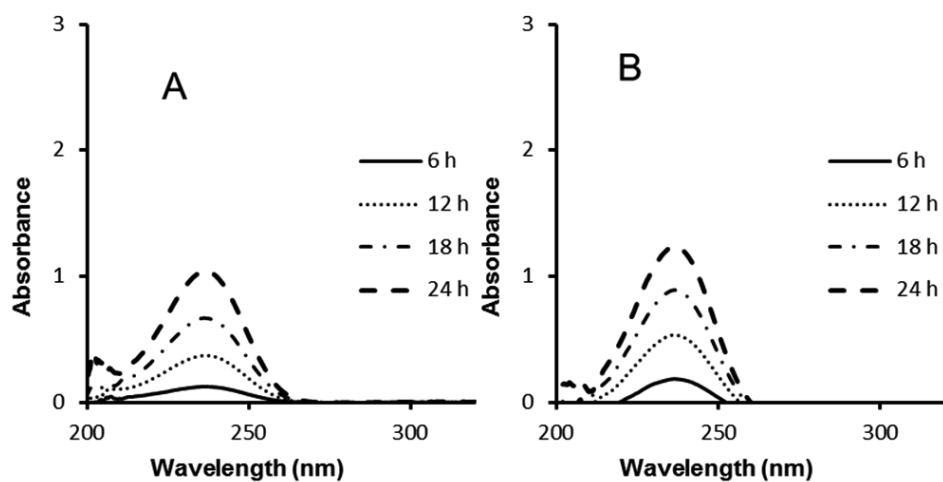


Figure S13. UV spectra recorded after incubation of **14** (16.7 mM) in 20 mM NaH_2PO_4 buffer at pH 7.3 with 0.045 mM A) recombinant WT 4-OT and B) synthetic WT 4-OT ($t = 6, 12, 18$ and 24 h).

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